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14. ABSTRACT
The surface molecules of bacteria contribute significantly to the host's response to both pathogens and commensals. However, the technology available to track these molecules in host cells and tissues remains primitive. To address this limitation, we have developed an expanded metabolic labeling approach that chemically tags lipopolysaccharide, capsular polysaccharide, and peptidoglycan simultaneously in live anaerobic commensal bacteria. This technology enabled us to track the entry of differentially labeled surface molecules from live, luminal bacteria into specific host intestinal immune cells and their subsequent degradation in host phagocytes. Notably, this approach also enabled live imaging of the tagged bacterial surface molecules from endogenous commensals in the intestine of the living murine host. These tools will make it possible for investigators to decipher the role of specific bacterial surface molecules in host response.

15. SUBJECT TERMS
Bioorthogonal click-chemistry, bacterial cell wall, bacterial outer membrane, peptidoglycan, lipopolysaccharide, endotoxin, capsular polysaccharide, inflammatory bowel disease, microbiome, microbiota, carbohydrate chemistry, fluorescent microscopy, 2-photon microscopy, confocal microscopy

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1. INTRODUCTION:

In this proposal, we will apply and expand our bioorthogonal click-chemistry strategy to simultaneously label an unprecedented three components of the bacterial cell wall: PSA, lipopolysaccharide (LPS), and peptidoglycan (PGN). With this approach, we will be able to track a TLR2 ligand (PSA), a TLR4 ligand (LPS), and a ligand for NLR signaling (PGN) in living bacteria and their host and to follow these molecules into specific host immune cells in order to define the pathways by which protective or inflammatory responses develop. This methodology will also allow us to visualize the spatial and temporal changes in bacterial product acquisition within diseased tissue. Beyond adding to the basic knowledge of host-commensal communication, an understanding of microbial sensing in the gut will allow us to design new treatments for IBD patients. Our long-term objective is to use fluorescently labeled commensal bacteria as a less invasive tool for the study of IBD in patients. This approach leverages the Kasper lab's expertise in commensal microbiology and carbohydrate chemistry with the von Andrian lab's skills in fluorescence microscopy and immunology to develop a new interdisciplinary platform for imaging and tracing bacterial products in the gut.

2. KEYWORDS:

Bioorthogonal click-chemistry, cell wall, outer membrane, peptidoglycan, lipopolysaccharide, endotoxin, capsular polysaccharide, IBD, microbiome, microbiota, carbohydrate chemistry, fluorescent, 2-photon, confocal microscopy

3. ACCOMPLISHMENTS:

There were three specific aims in our proposal.

Aim 1. Incorporate non-natural sugars and amino acids into lipopolysaccharide, capsular polysaccharide, and peptidoglycan for live bacterial-cell labeling.

Aim 2. Use click labeling to elucidate the acquisition and distribution of commensal material *in vitro* and in host intestinal tissue.

Aim 3. Use live intravital microscopy to monitor changes in bacterial-component acquisition during chemical and pathogen-induced colitis.

We have made significant progress on all three specific aims as summarized below. The Kasper lab has done the chemistry, microbiology and worked with the von Andrian lab on the immunology and microscopy. All work was done between the two labs at Harvard Medical School.

We have published a major original research article in *Nature Microbiology (2017; vol. 2, 17099)* entitled, "Illuminating vital surface molecules of symbionts in health and disease." We used a combination of metabolic labeling and bioorthogonal click chemistry (that is reactions performed in living organisms) to specifically tag up to 3 prominent surface immunomodulatory macromolecules – peptidoglycan, lipopolysaccharide and capsular polysaccharide – either simultaneously or individually in live anaerobic commensal bacteria. Importantly, the peptidoglycan labeling enables, for the first time, the specific labeling of live, endogenous anaerobic bacteria within the mammalian host. This approach has allowed us to image and track the path of labeled surface molecules from live, luminal bacteria into specific intestinal immune cells in the living murine host during health and disease. The chemical labeling of three specific macromolecules within a live organism offers the potential for in depth visualization of host-pathogen interactions. These specific studies are presented in the manuscript as Appendix A.

There have been four postdoctoral fellows and one technician working on this project and the work has afforded significant training in solving the chemical, immunologic, and microbiologic aims of this grant. Three fellows from the Kasper lab do the chemistry, microbiology and work with the two fellows from the von Andrian lab on the immunology and microscopy. The technician has been responsible for the synthesis of chemical reagents for fluorescent labeling. Teaching has come from several sources including direction and advice from PI's and senior lab members, conferences, and regular lab meetings.

Dissemination of knowledge to the community is in the form of a major research article to be found in Appendix A.

We will continue to advance the biologic insights gained using the technological advances reported in our manuscript. We have already met the great majority of the technological goals in all three specific aims. We will now apply these advances to understand disease mechanisms and interactions between immune cells and microbes in inflammatory bowel disease and in colon cancer. Our basic approach is outlined as found in the manuscript (Figure 2). In Figure 2 we have shown that in inflammatory bowel disease peptidoglycan can be followed into specific immune cells by HADA labeling. This provides a powerful tool for understanding which bacterial molecules impact of specific subsets of immune cells during inflammation.

4. IMPACT:

The techniques we have developed provide important tools that will enable research at the interface of host microbe interactions during disease. An understanding of these interactions provides key insights into potential avenues for therapeutic intervention.

These chemical techniques are also a potential method for delivery of antibiotics directly to the target in the bacterial cell. We are exploring the possibility of commercial impact of this approach.

5. CHANGES/PROBLEMS:

Nothing to Report

6. PRODUCTS:

Published

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Acknowledgement of federal support: Yes

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

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Project Role: Principal Investigator
No change

Name: **Ulrich von Andrian**
Project Role: Principal Investigator
No change

Name: **Naama Geva Zatorsky**
Project Role: Research Fellow
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Name: **Xinyang Song**
Project Role: Research Fellow
Nearest person month worked: 6
Contribution to Project: *Dr. Song is a postdoctoral fellow working on this project and is directly involved with the immunologic component of this study.*

Name: **Meng Wu**
Project Role: Research Fellow
Nearest person month worked: 6
Contribution to Project: *Dr. Wu is a postdoctoral fellow working on creating bacterial mutants to specifically explore mechanisms of organism immune interaction.*

Name: **Kailyn Stefan**
Project Role: Graduate Student
No Change

Name: **Jason Daugherty**
Project Role: Technician
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Contribution to Project: *Mr. Daugherty is a technician responsible for synthesis of chemical reagents required for bio-orthogonal chemistry.*

Name: **Bella Printseva**
Project Role: Research Administrator
No Change

Name: **Munir Mosaheb**
Project Role: Research Fellow
Nearest person month worked: 5
Contribution to Project: *Dr. Mosaheb is a postdoctoral fellow working on this project. He is responsible for conducting intravital two photon microscopic studies.*

Name: **Pavel Hanc**
Project Role: Research Fellow
Nearest person month worked: 6
Contribution to Project: *Dr. Hanc is a postdoctoral fellow working on this project. He is responsible for conducting experiments using two photon intravital microscopy in the pathogen-induced colitis model.*

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9. APPENDICES:

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LETTERS

PUBLISHED: 26 JUNE 2017 | VOLUME: 2 | ARTICLE NUMBER: 17099

Illuminating vital surface molecules of symbionts in health and disease

Jason E. Hudak[1], David Alvarez[1], Ashwin Skelly[1], Ulrich H. von Andrian[1,2] and Dennis L. Kasper[1]*

The immunomodulatory surface molecules of commensal and pathogenic bacteria are critical to microorganisms' survival and the host's response[1,2]. Recent studies have highlighted the unique and important responses elicited by commensal-derived surface macromolecules[3–5]. However, the technology available to track these molecules in host cells and tissues remains primitive. We report, here, an interdisciplinary approach that uses metabolic labelling combined with bioorthogonal click chemistry (that is, reactions performed in living organisms)[6] to specifically tag up to three prominent surface immunomodulatory macromolecules—peptidoglycan, lipopolysaccharide and capsular polysaccharide—either simultaneously or individually in live anaerobic commensal bacteria. Importantly, the peptidoglycan labelling enables, for the first time, the specific labelling of live endogenous, anaerobic bacteria within the mammalian host. This approach has allowed us to image and track the path of labelled surface molecules from live, luminal bacteria into specific intestinal immune cells in the living murine host during health and disease. The chemical labelling of three specific macromolecules within a live organism offers the potential for in-depth visualization of host–pathogen interactions.

Current methods to study commensal–host interactions *in situ*, such as fluorescence *in situ* hybridization, remain limited in scope. Despite significant advances in updating this technology[7,8], it still suffers from many drawbacks[9]. Chemical-based probes have been used to image and track bacterial components that are otherwise recalcitrant to conventional genetic tagging methods[10,11]. Furthermore, the predominantly anaerobic environment of the intestinal lumen presents an additional hurdle to genetically encoded tags such as green fluorescent protein (GFP), which require oxygen to mature. In this vein, we previously reported a method to tag and trace the capsular polysaccharides (CPSs) of various live commensal bacteria in cells and animal hosts[12]. This approach uses the metabolic incorporation of a non-natural sugar, *N*-azidoacetylgalactosamine (GalNAz)[13], into bacterial CPS to tag and track the bacterium and its CPS. However, fewer than 50% of anaerobic microorganisms tested could be reliably labelled with this approach. Therefore, we sought to expand and improve this method to (1) label a larger subset of commensals that did not incorporate GalNAz and (2) extend the tagged targets to other immunomodulatory surface molecules, especially more common bacterial molecules.

For our first target, the peptidoglycan (PGN) component of bacteria seemed an obvious choice as it is a highly conserved structural feature of most bacterial phyla. PGN is sensed by the innate nucleotide-binding oligomerization domain-like receptors (NOD-like receptors) in mammalian cells, and the strong link of mutations in these receptors to inflammatory bowel disease (IBD) highlights their importance in maintaining healthy commensal–host interactions[14,15]. The promiscuity of PGN biosynthesis was recently exploited in developing a method to install non-natural fluorescent D-amino acids into bacterial PGN (Fig. 1a)[16,17]. Because cells use only a defined set of L-amino acids for protein synthesis, only the provided D-amino acids can specifically label the PGN. We wanted to determine whether this approach could be used to label and track anaerobic commensal bacteria both *in vitro* and *in vivo*.

Incubation of anaerobic cultures with the fluorescent D-amino acid hydroxycoumarin amino-D-alanine (HADA) resulted in the successful labelling of a wide range of anaerobic and facultative commensal bacteria, including *Bacteroides fragilis*, *Bacteroides vulgatus*, *Parabacteroides merdae*, *Clostridium clostridioforme*, *Clostridium ramosum*, *Enterococcus faecalis*, *Escherichia coli* and *Bifidobacterium adolescentis* (Fig. 1a,b and Supplementary Fig. 1a). As a control, we incubated the bacteria with the L-enantiomer of the fluorescent amino acid referred to as HALA, which does not incorporate into PGN or other macromolecules. The minimal background we observed supported the specific labelling of the PGN layer by D-amino acid incorporation. This labelling is both time- and concentration-dependent, as previously reported (Supplementary Fig. 1b)[16], although overnight incubation with HADA was optimal, as previously reported for labelling with GalNAz[12].

Given the success of *in vitro* PGN labelling, we examined whether the labelled bacteria could be imaged and traced within the natural niche of the host intestine. *B. adolescentis* and *E. faecalis* were labelled as described and administered to mice via oral gavage and direct intestinal injection, respectively. The bacteria retained the PGN label and were successfully imaged in tissue sections of the small intestine and colon (Fig. 1c and Supplementary Fig. 2a). *E. faecalis* in particular was found close to the tissue in the proximal colon and in the vicinity of $CD11c^+$ antigen-presenting cells (APCs, Fig. 1c). We also synthesized fluorescein-D-lysine (FDL)[16] and labelled *C. clostridioforme* in culture. Imaging of this organism in the murine colon (Supplementary Fig. 2b,c) demonstrated the capacity for multicolour labelling.

Because exogenous D-amino acids are not used by mammalian cells[10], we reasoned that feeding conventionally raised mice the fluorescent D-amino acid might allow selective labelling of endogenous bacteria. Confocal imaging of tissue histology slices from HADA-gavaged specific pathogen-free (SPF) mice showed robust labelling of the commensal bacteria already present in the lumen of the small intestine and the colon 2 and 4 h, respectively, after gavage (Fig. 1d). By contrast, the HALA-gavaged controls showed little background. This approach overcomes a significant methodological obstacle by providing the ability to specifically label the endogenous microbiota within a living host.

Further examination of the luminal contents demonstrated that the bacteria in the small intestine were quickly labelled (within 45 min) after HADA administration, but this signal was washed out within 3 h (Fig. 1e). This result is in accord with previous reports that small-intestinal niches are less populated and rapidly

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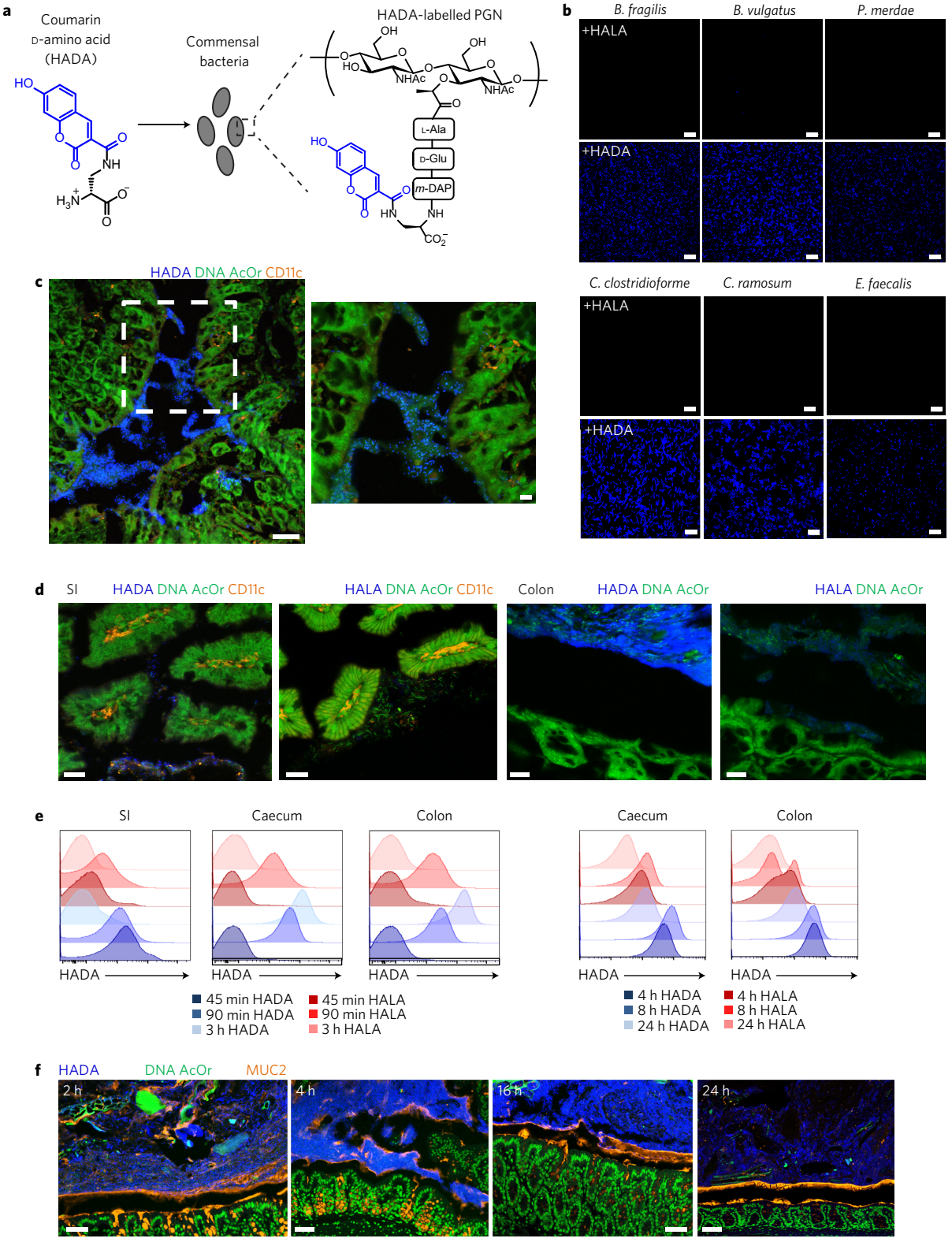

Figure 1 | Fluorescent D-amino acid labels PGN in commensal bacteria. a, Schematic of metabolic labelling of the PGN of commensal bacteria with fluorescent D-amino acid derivatives. Addition of the fluorescent coumarin moiety is highlighted in blue. **b**, Images of various commensal bacteria grown overnight in medium with a fluorescent amino acid: HADA or (as a negative control) HALA. Scale bars, 10 μm. **c**, Confocal image and expanded region (right) of frozen, fixed tissue slice of mouse colon 1 h after injection of HADA-labelled *E. faecalis*. AcOr, acridine orange. Scale bars, 50 μm (left) and 10 μm (right). **d**, Confocal images of Carnoy's-fixed, paraffin-embedded tissue slices from mice 2 h (small intestine (SI), left) or 4 h (colon, right) after oral gavage with HADA or (as a negative control) HALA. Scale bars, 20 μm. **e**, Flow cytometry histogram plots of luminal contents from mice given HADA or HALA orally demonstrate decay of fluorescent signal over time. **f**, Confocal images of Carnoy's-fixed, paraffin-embedded tissue slices from mice given HADA orally. The mice were killed and tissue was fixed at the specified times after oral gavage. The images show significant retention of fluorescent signal over 24 h. MUC2, mucin 2. Scale bars, 50 μm. Data in **c**–**f** are representative of at least three independent experiments.

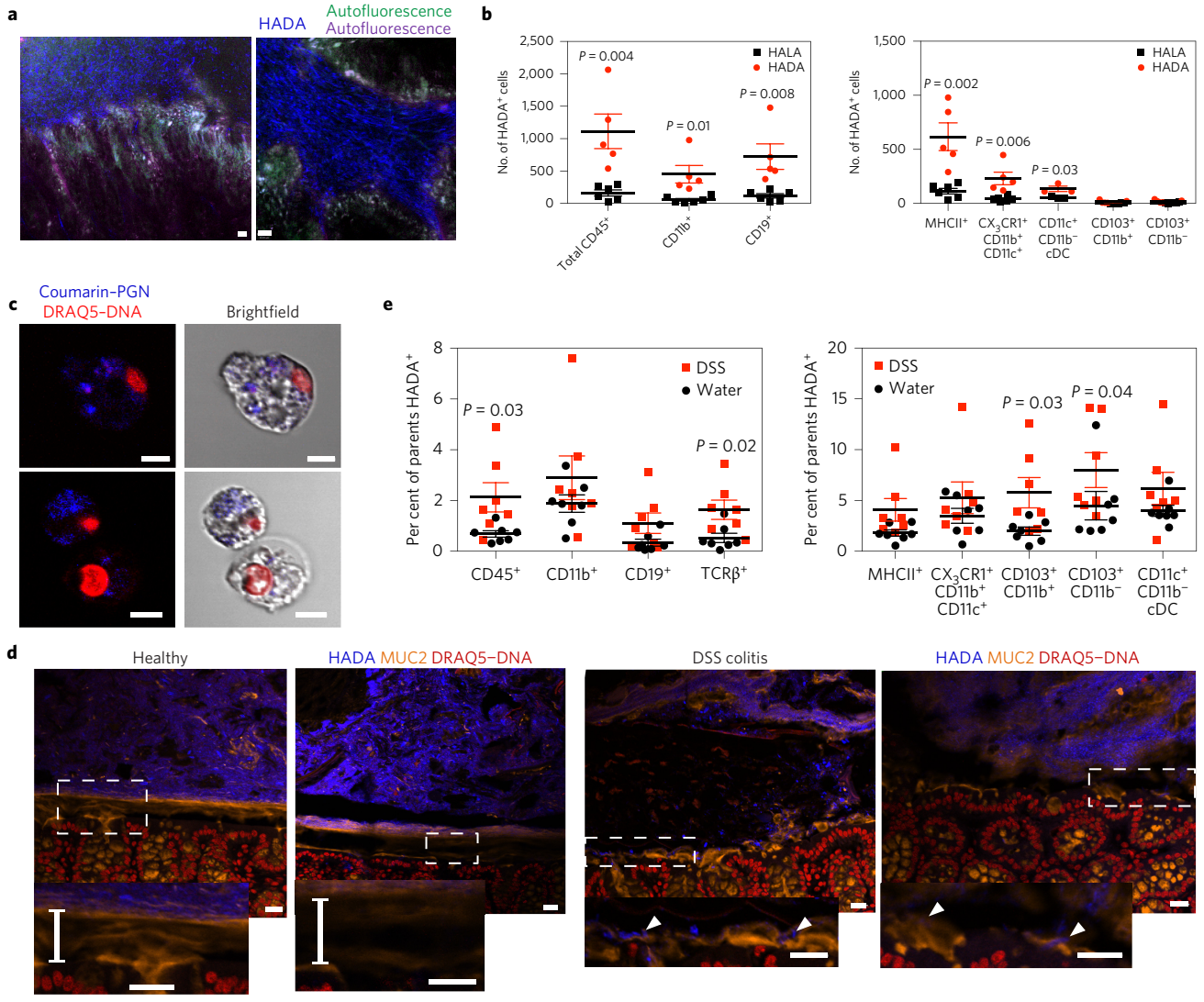

Figure 2 | Use of HADA-labelled PGN to track live commensals in the host. a, Representative live images from intravital two-photon microscopy of the murine colon 16 h after administration of HADA. Mice were anaesthetized and intestinal loops were surgically extracted and mounted for imaging. Purple and green represent tissue autofluorescence (Supplementary Videos 1 and 2). Scale bars, 10 μm. **b**, Numbers of colonic lymphocytes bearing a HADA$^+$ signal 4 h after oral gavage of HADA. Data are presented as mean ± s.e.m. values for biological replicates of $n = 5$ mice (P values calculated by paired Student's t-test). **c**, Representative confocal images of live CD11c$^+$ (top) or CD11c$^+$CX$_3$CR1$^+$ (bottom) mononuclear phagocytes (sorted for a HADA$^+$ signal) bearing HADA-labelled bacteria/PGN. Scale bars, 5 μm. DRAQ5, red DNA dye. **d**, Representative images of Carnoy's-fixed, paraffin-embedded tissue slices obtained 4 h after oral gavage of HADA from mice given normal drinking water (left) or drinking water containing 3% DSS (right) for the preceding 4 days. The damaged mucosal layer and breach with endogenous bacteria are evident in the DSS-induced colitic colons. Bars show that mucus thickness is ~50 μm in healthy mice and arrowheads point at bacteria at the epithelial layer in DSS-treated mouse colons. Scale bars, 20 μm. **e**, Percentages of the parent subpopulation of colonic lymphocytes bearing a HADA$^+$ signal 4 h after oral gavage with HADA in healthy mice and mice with DSS-induced colitis. Data are presented as mean ± s.e.m. values for biological replicates of $n = 7$ mice (P values calculated by paired Student's t-test). Data in **a**–**e** are representative of at least three independent experiments.

flush luminal content[18]. Correspondingly, the caecum and colon showed maximal labelling of endogenous bacteria within 3–8 h (Fig. 1e). The high signal from HALA samples at earlier time points suggests that several hours are required for the excess label to flush out naturally. Surprisingly, commensal bacteria remained significantly labelled up to 24 h after initial HADA administration by oral gavage (Fig. 1e,f and Supplementary Figs 3a,b and 4). This result suggests that, despite clearance of the PGN label within 6–8 h in rich media *in vitro* (Supplementary Fig. 3c), the label *in vivo* is retained much longer, perhaps because of slow growth rates in the normal gut. Repetition in germ-free (GF) mice showed minimal background signal, again suggesting that the observed fluorescence staining is dependent on PGN from endogenous bacteria (Supplementary Fig. 5a,b).

Because our approach labels living bacteria, we also used multiphoton intravital microscopy to view the dynamics of the host–microbiota interaction in real time[19]. Our results showed robust labelling of the commensal microorganisms in the small and large intestines (Fig. 2a and Supplementary Fig. 6). Videos of these dynamics highlight the ability to view endogenous bacteria moving within the lumen and adhering to the mucosal surface of the epithelial barrier (Supplementary Videos 1 and 2).

If the PGN of HADA-labelled bacteria could be imaged and traced in the lumen, then this approach would have the potential to track PGN from live commensals into specific intestinal cells. Several studies have investigated how free luminal protein or pathogens gain access to gut-associated lymphoid tissue (GALT); however, much less is known about bacterial structural-component

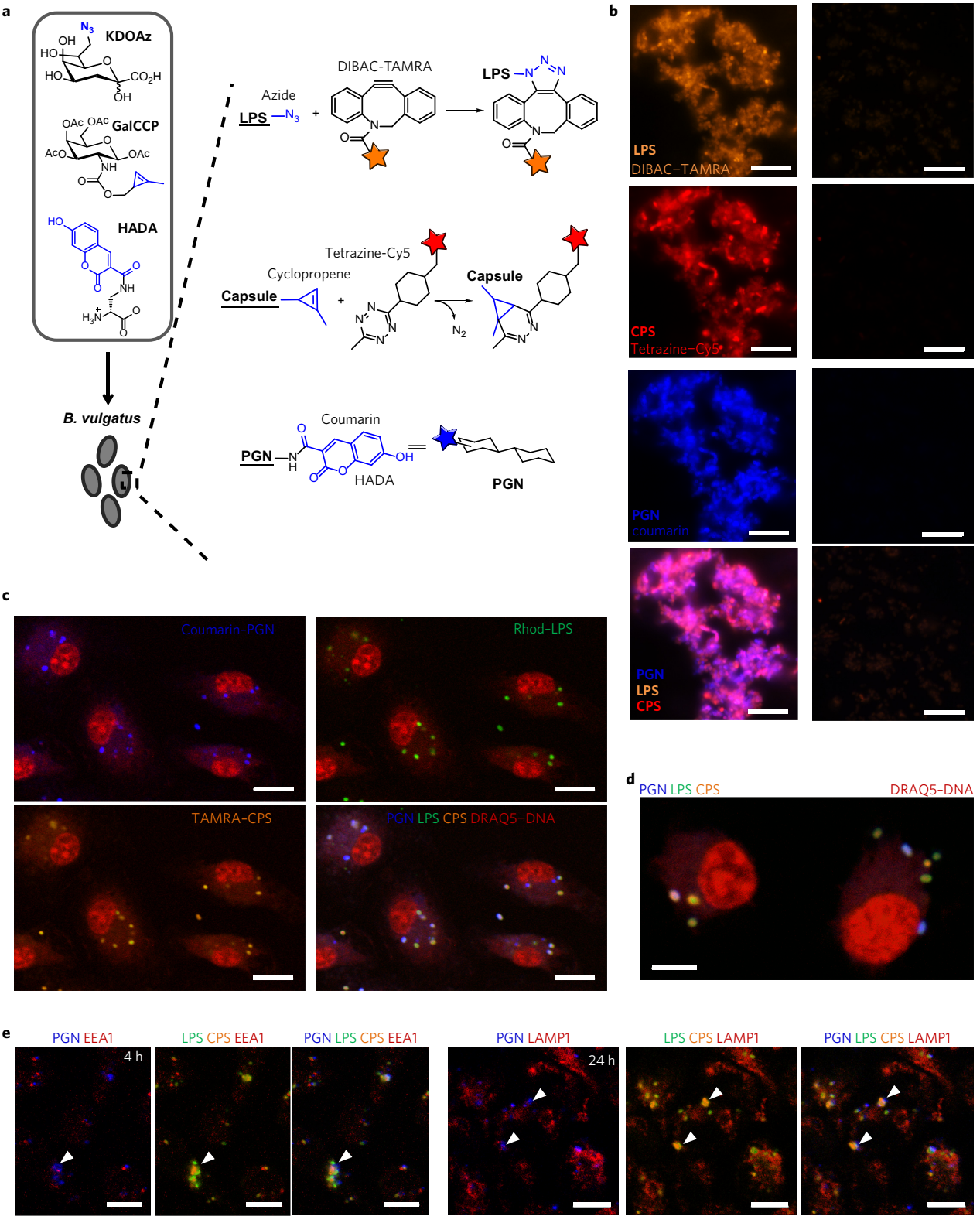

Figure 3 | Simultaneous labelling of three cell surface molecules in commensal bacteria. a, Schematic of the metabolic labelling approach using the non-natural metabolites KDOAz (LPS), GalCCP (CPS) and HADA (PGN) to simultaneously label three cell surface macromolecules in the commensal bacterium *B. vulgatus*. The reactive or fluorescent moieties are highlighted in blue. DIBAC-TAMRA, dibenzo-aza-cyclooctyne-carboxytetramethylrhodamine; Cy5, cyanine-5. **b**, Images of *B. vulgatus* grown overnight in medium with KDOAz, GalCCP and HADA (left) or, as a negative control, with GalNAc and HALA (right) and reacted with DIBAC-TAMRA and tetrazine-Cy5. Scale bars, 10 μm. **c**, Confocal images of fixed bone-marrow-derived macrophages 1 h after inoculation of three-component-labelled *B. vulgatus*. Rhod, rhodamine green; DRAQ5, red DNA dye. Scale bars, 5 μm. **d**, Zoomed confocal image of bone-marrow-derived macrophages bearing three-component-labelled *B. vulgatus*. Scale bar, 5 μm. **e**, Representative images of J775A.1 murine macrophages 4 h and 24 h after inoculation of three-component-labelled *B. vulgatus*. Arrows highlight where PGN is seen separate from colocalized CPS/LPS. Scale bars, 10 μm. Data in **b**–**e** are representative of at least three independent experiments with $n = 3$ technical replicates.

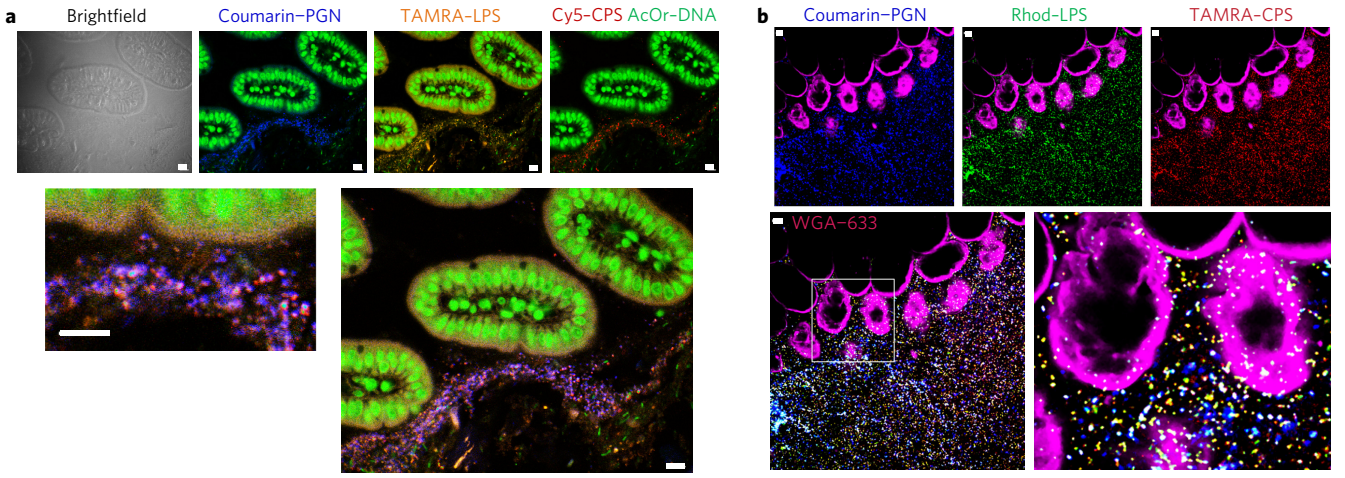

Figure 4 | Simultaneous labelling of three cell surface molecules in commensal bacteria. a, Confocal image of Carnoy's-fixed, paraffin-embedded tissue slices obtained from mice 1 h after intestinal injection of labelled *B. vulgatus*. Zoomed image (lower left) is shown to highlight the interaction with the host epithelial layer. Scale bars, 10 μm. **b**, Representative live images from intravital two-photon microscopy of the murine small intestine after direct inoculation with three-component-labelled *B. vulgatus*. Purple represents lectin staining of the epithelium with WGA-633 (Supplementary Videos 3 and 4). Scale bars, 10 μm. Data are representative of at least three independent experiments with $n = 3$ mice per experiment.

transport, especially in commensals[20,21]. Flow cytometry analysis of colonic lamina propria cells from mice receiving HADA or HALA revealed a significant portion of $CD45^+$ lymphocytes containing HADA fluorescence (Fig. 2b and Supplementary Fig. 7). Among these $HADA^+$ lymphocytes, $CD11b^+$ phagocytes and $CD19^+$ B cells predominated. This large number of $HADA^+CD19^+$ B cells was a surprise. Although dendritic cells (DCs) are known to stimulate B cells in the GALT[22], few studies have cited such high levels of direct commensal association with resident B cells.

Aside from $CD19^+$ B cells, the APCs in the $MHCII^+HADA^+$ population of the colon consisted mainly of $CX_3CR1^+CD11c^+CD11b^+$ macrophages and conventional $CD11c^+CD11b^-$ DCs. CX_3CR1^+ macrophages are known to directly sample luminal bacteria and to play key roles in phagocytosis and antigen presentation in the lamina propria[21,23]. $CD103^+$ cells are the main DCs to migrate to the mesenteric lymph nodes[21]. However, we did not see a significant population of $HADA^+CD103^+$ DCs in the colonic lamina propria (Fig. 2b). This result may be due to the small numbers of this cell type in the lamina propria or may highlight the limited ability of these cells to directly acquire luminal bacteria. To confirm that $HADA^+$ cells did indeed contain PGN or PGN-labelled bacteria, we sorted and imaged the $CD11c^+CX_3CR1^+$ and $CD11c^+CX_3CR1^-$ cells containing HADA fluorescence (Fig. 2c and Supplementary Fig. 8).

Sensing of PGN by the host immune system plays a role in the development and progression of IBD[14]. We used the D-amino acid metabolic labelling approach in a mouse model of colitis to track and image the PGN of commensals during disease-associated inflammation. As seen in Fig. 2d, the mucosal layer within the colon was greatly diminished and this change allowed direct interaction of the labelled endogenous bacteria with the colonic epithelium, a finding previously described in IBD[24,25]. This interaction correlated with an increase in $HADA^+CD45^+$ lymphocytes in the lamina propria (Fig. 2e). Of these lymphocytes, T-cell receptor β (TCRβ)-positive T cells and $CD103^+$ DCs were significantly associated with bacteria/bacterial PGN in the colitic versus healthy host, a finding that further supports a key role for these cells in microbial influences on colitis[25].

The utility of the PGN D-amino acid labelling approach impelled us to seek yet more surface molecules that could be tagged to trace commensals *in vivo*. Recently, an azide-containing 2-keto-3-deoxy-D-mannooctanoic acid (KDO) derivative, 8-azido-8-deoxy-KDO (KDOAz), was shown to incorporate into the lipopolysaccharide (LPS) of *E. coli* (Supplementary Fig. 9a)[26]. Once incorporated, the azido group can be labelled by reaction with a fluorescent-stained alkyne to form a stable, covalent triazole[6]. We anticipated that this approach would be broadly applicable because the LPS of many other Gram-negative bacteria consists of an acylated lipid A with a KDO linker in its core oligosaccharide[27]. As confirmed by microscopy, flow cytometry and gel electrophoresis, KDOAz incorporated into LPS and labelled a wide variety of Gram-negative intestinal resident bacteria, including *E. coli*, *Klebsiella*, *B. vulgatus*, *Bacteroides uniformis*, *Bacteroides eggerthii* and *Fusobacterium necrophorum* (Supplementary Figs 9b,c and 10).

At this point we had successfully shown labelling of CPS, PGN and LPS on a variety of commensal bacteria. Could we then label all three of these immunomodulatory surface components simultaneously on live commensals and thereby overcome a significant previous obstacle—the simultaneous metabolic labelling of multiple distinct molecules in one organism? GalNAz or KDOAz labelling relies on the same azide chemical handle for fluorophore attachment, so we devised an orthogonal labelling approach for CPS, using a GalNAc derivative that contains a cyclopropene, *N*-cyclopropenyl galactosaminyl carbamate (GalCCP) (Fig. 3a)[28]. Like azides, cyclopropenes are small and biologically inert functional groups, but they react selectively with tetrazines and at the same time as the azide–alkyne reaction[29]. Thus, LPS and CPS could conceivably be labelled simultaneously with different fluorophores.

We tested our three-component labelling scheme on the commensal *B. vulgatus* because it performed well with the individual components. By flow cytometry and microscopy, we documented distinct labelling of all three components, with minimal background in controls incubated with HALA and GalNAc (Fig. 3b and Supplementary Fig. 11a). The distinct labelling of the LPS and CPS components was confirmed by SDS–PAGE, by which the high-molecular-weight (M_w) CPS for tetrazine–GalCCP can be distinguished from the lower-M_w LPS ladder for cyclooctyne–KDOAz (Supplementary Fig. 11b).

A main appeal of this approach is its ability to track how individual commensal components are broken down and sensed by immune cells. To test this utility, we incubated three-component-labelled *B. vulgatus* with the murine macrophage cell line J755A.1 or bone-marrow-derived macrophages. At early time points, all

three components could be seen distinctly in association with phagocytosed bacteria in the macrophages (Fig. 3c,d and Supplementary Fig. 12a,b). Within 4–24 h, the components were often seen to separate into distinct areas: LPS and CPS were located in large encapsulated endosomes, while the HADA-PGN signal was frequently found in punctate areas around these lysosomal LAMP-1$^+$ compartments (Fig. 3e, indicated by arrowheads). In cells treated with bafilomycin, which halts endosome acidification, these large LPS$^+$CPS$^+$ compartments could not be found, a result implying that this phenomenon is a product of cell-mediated bacterial degradation (Supplementary Fig. 12c).

The three-component-labelled bacteria were also introduced and imaged within the intestinal lumen of the murine host. This experiment was performed (1) on fixed tissue sections from mice given labelled *B. vulgatus* by direct intestinal injection and (2) with two-photon intravital microscopy on surgically exposed intestinal loops in live mice given labelled *B. vulgatus* by direct application (Fig. 4a,b and Supplementary Fig. 13a,b). Live videos (Supplementary Videos 3 and 4) highlight the ability to track each component as the bacteria traverse the lumen and interact with host epithelial cells.

The metabolic labelling and click chemistry approach we present here allows, for the first time, simultaneous labelling of three bacterial components: CPS, LPS and PGN. Because most of the mammalian microbiota resides in the intestine, deciphering how and when these bacterial molecules are obtained by the host is especially important in understanding the progression of intestinal diseases, including IBD and colorectal cancer[30]. The ability to track these inflammatory (and anti-inflammatory) inducers will enable the study of where they accumulate, which cell types they encounter in GALT, and, ultimately, whether differences in their localization correlate with disease.

Methods

Mice. Male and female 6- to 8-week-old C57BL/6 mice were purchased from Jackson Laboratory. Mice were housed in SPF conditions with food and water *ad libitum*. GF C57BL/6 mice were bred and maintained in sterile vinyl isolators in the animal facility at Harvard Medical School and were provided with sterile food (LabDiets 5K67), water and bedding. All experiments were conducted in accordance with the guidelines of the US National Institutes of Health and approved by the Harvard Medical Area Standing Committee on Animals.

Bacteria and growth media. The following bacterial species were used: *Bacteroides fragilis* (NCTC9343), *Bacteroides eggerthii* (DSM 20697), *Bacteroides uniformis* (ATCC8452), *Bacteroides vulgatus* (ATCC8482), *Parabacteroides merdae* (CL03T12C32), *Fusobacterium necrophorum* (AO43), *Clostridium clostridioforme* (2_1_49FAA), *Clostridium ramosum* (AO31), *Escherichia coli* (K12), *Bifidobacterium adolescentis* (L2-32), *Klebsiella* spp. (4_1_44FAA) and *Enterococcus faecalis* (TX0104). All bacteria were grown in a basal peptone-yeast broth containing (per litre) 5 g of yeast extract, 20 g of protease peptone, 5 g of NaCl, 5 mg of hemin, 0.5 mg of vitamin K1 and 5 g of K_2HPO_4. Hemin, vitamin K1 and K_2HPO_4 were added through a filter after the basal medium had been autoclaved. All non-natural metabolites for metabolic labelling were synthesized in house (for details see Supplementary Note 1).

Metabolic labelling and click chemistry of bacteria. Bacteria were inoculated from a plate culture and grown overnight at 37 °C under anaerobic conditions (80% N_2, 10% H_2, 10% CO_2) in an anaerobic chamber in basal peptone-yeast broth with HADA or HALA (0.8 mM), KDOAz (5 mM) and/or GalCCP (250 μM). Bacteria were pelleted at 5,000*g* and washed twice in PBS and once in PBS supplemented with 1% BSA. For the subsequent click reactions with KDOAz and GalCCP, the final pellet was resuspended in one-tenth the original culture volume (for example, 100 μl for a 1 ml culture) of 1% BSA in PBS with 5 μM DIBAC-Rhod, DIBAC-Cy5 or DIBAC-TAMRA (Click Chemistry Tools) for reaction with azide or 5 μM tetrazine-TAMRA/tetrazine-Cy5 (Click Chemistry Tools) for reaction with GalCCP. The suspension was incubated with agitation for 1 h at 37 °C, after which the bacteria were pelleted and washed twice with 1% BSA in PBS and once in PBS. For three-component labelling, the bacteria were first reacted with 5 μM DIBAC for 1 h at 37 °C, washed once with PBS and then reacted with 5 μM tetrazine for 1 h at 37 °C. After the final wash, cells were resuspended in PBS and were administered to mice or fixed in 1% formalin and mounted for microscopy or run on flow cytometry. To monitor label decay, bacteria were resuspended in basal broth and aliquots were removed and fixed in 1% formalin at specified time points.

Tissue culture studies and immunofluorescent microscopy. *B. vulgatus* was grown overnight in basal peptone-yeast broth with HADA (0.8 mM), KDOAz (5 mM) and GalCCP (250 μM) to an optical density at 600 nm (OD_{600}) of ~0.6. As a control, bacteria were grown in HALA (0.8 mM) and GalNAc (250 μM). The bacteria were labelled with tetrazine-Cy5 and DIBAC-TAMRA or tetrazine-TAMRA and DIBAC-Rhod as described in the previous section. C57BL/6 primary-bone marrow-derived macrophages or J775A.1 murine macrophages were isolated from mouse bone marrow or received directly from the ATCC, respectively, without further authentication or mycoplasma testing. A total of 5×10^5 macrophage cells were seeded onto 18 mm coverslips in 12-well plates and grown to confluency under humid conditions in an atmosphere of 5% CO_2 at 37 °C in RPMI medium supplemented with 10% fetal bovine serum (FBS), 1% sodium pyruvate, 1% nonessential amino acids, 1% penicillin–streptomycin, 0.15% sodium bicarbonate and 2 mM L-glutamine. Cells were washed with PBS and each well was filled with RPMI medium containing no penicillin–streptomycin. A 10 μl volume of labelled bacterial cells (multiplicity of infection of ~10) was added to each well and spun at 300*g* for 5 min. For some studies, 0.1 μM bafilomycin (Enzo Life Sciences) was added from a DMSO stock to inhibit lysosome maturation. After incubation for 30 min in 5% CO_2 at 37 °C, the medium was removed and coverslips were washed with sterile RPMI containing no penicillin–streptomycin and left in RPMI. Coverslips were removed from the plates at 1, 4 and 24 h, washed with PBS, and fixed for 20 min in 4% paraformaldehyde in PBS. Cells were blocked and permeabilized by incubation with 10% FBS, 1% BSA and 0.1% Triton X-100 in PBS for 1 h, washed twice with Tris-buffered saline with 0.1% Tween (TBST), and incubated with antibodies to LAMP-1 (sc-19992, clone 1D4B, lot H1612, Santa Cruz Biotechnology) or EEA-1 (sc-6415, clone N-19, lot B1813, Santa Cruz Biotechnology) (1:200 in 2% BSA/PBS) for 16 h (protected from light) at 4 °C. Nuclear staining was performed with acridine orange (Biotium) or DRAQ5 (BioLegend) (1 μg ml^{-1}) for 10 min. Coverslips were washed three times with TBST, mounted on glass slides with Mowiol medium, and viewed on an Olympus Fluoview BX50WI inverted confocal microscope. All images were analysed with Fluoview Viewer Software and prepared in Adobe Photoshop.

Tracking of labelled bacteria in the murine host. For tracking of endogenous bacteria, C57BL/6 mice received 250 μl of 3 mM HADA/HALA in PBS by oral gavage. At specified time points, mice were euthanized with CO_2 and the intestinal tissue and mesenteric lymph nodes were removed. In most experiments, the selected time point was 2 h after gavage for the small intestine and 4 h after gavage for the colon. For tracking of exogenously labelled bacteria, organisms were grown in basal medium containing metabolic labels as described under 'Bacteria and growth media'. Approximately 10^8–10^9 organisms in 100 μl of PBS were directly injected into the lumen of the ileum or proximal colon of live SPF C57BL/6 mice anaesthetized under isofluorane. At 1 h after injection, mice were euthanized with CO_2 and the intestine and mesenteric lymph nodes were removed. In the dextran sodium sulfate (DSS)-induced colitis model, mice received 3% DSS in the drinking water as previously described[31] and were monitored daily. Colonic tissue was collected from mice on day 4 after administration of DSS (at the onset of intestinal inflammation)[31] and lymphocytes were isolated.

For fluorescence imaging, intestinal tissue was fixed overnight in Carnoy's fixative (60% methanol, 30% chloroform, 10% acetic acid) and submitted to the Harvard Cancer Center Research Pathology Core for paraffin embedding and sectioning. Paraffin was removed by two incubations in xylene substitute (ThermoFisher) for 10 min at 37 °C followed by rehydration with sequential incubation in 100, 95, 70 and 50% ethanol and then in distilled water. Alternatively, tissue was fixed in 4% formalin/DMEM for 4 h at 4 °C, embedded in OCT compound (Tissue-Tek), frozen into tissue moulds and later sectioned at a thickness of ~12 μm with a cryostat. The tissue sections were blocked and permeabilized by incubation with 10% FBA, 1% BSA and 0.1% Triton X-100 in PBS for 1 h, washed twice with TBST, and incubated with antibodies to CD11c (clone N418, Santa Cruz Biotechnology) or MUC2 (clone H-300, lot F2314, Santa Cruz Biotechnology; 1:200 in 2% BSA/PBS) for 16 h (protected from light) at 4 °C. Secondary antibody to rabbit (AF568-goat IgG α-rabbit, Life Technologies; 1:1,000 in 2% BSA/PBS) was applied for 1 h at room temperature for MUC2 staining. Nuclear staining was performed with acridine orange (Biotium) or DRAQ5 (BioLegend) (1 μg ml^{-1}) for 10 min. Coverslips were washed three times with TBST and mounted on glass slides with Mowiol medium. Confocal images were acquired with an Olympus Fluoview BX50WI inverted confocal microscope. All images were analysed with Fluoview Viewer Software and prepared in Adobe Photoshop.

For analysis of single-cell isolates, intestinal tissues were cleaned and dissociated as previously described[32]. Intestinal luminal contents were flushed in 5 ml of PBS and fat was removed from tissue. For analysis of luminal bacteria, the flushed contents were allowed to settle and then filtered through nylon mesh, and material was pelleted and fixed overnight in 1% formalin/DMEM at 4 °C. The cleaned tissue was treated with RPMI containing 1 mM DTT, 2.5 mM EDTA and 1.5% FBS at 37 °C for 15 min to remove epithelial cells, minced and dissociated in collagenase solution (collagenase II (Gibco), 1.5 mg ml^{-1}; dispase (Gibco), 0.5 mg ml^{-1}; DNAse I (Worthington), 0.05 mg ml^{-1} and 1% FBS in RPMI) with constant stirring at 37 °C for 45 min. Single-cell suspensions were then filtered and washed in 5% FBS RPMI. Cell suspensions were filtered through nylon mesh and resuspended in

DMEM without phenol (Gibco/Life Technologies). This process was followed by staining on ice for 20 min (1:200 dilution) with fluorescently labelled antibodies to CD45 (30-F11), CD4 (GK1.5), CD11b (M1/70), CD11c (N418), CD19 (6D5), I-A/I-E (M5/114.15.2), CX_3CR1 (SAO11F11), CD103 (2E7) or TCRβ (H57-597), all from Biolegend. The fixable viability dye eFluor-780 (eBioscience) was used to gate out dead cells. The stained samples were immediately run or fixed in 1% formalin/DMEM overnight and run on flow cytometry (MacsQuant, Miltenyi or LSR-II, BD Biosciences) and analysed with FlowJo software. Cell suspensions were run on a FACSAria and sorted for $HADA^+$I-A/I-E^+CD11c^+ and for CX_3CR1^+ and CX_3CR1^-.

Intravital two-photon microscopy of the intestine. Intravital two-photon microscopy of mouse intestine was performed as previously described[12,33]. Mice were anaesthetized with ketamine–xylazine–acepromazine and positioned on a customized heated stage. A ~2 cm segment of intestine was exteriorized through the peritoneum, immobilized with tissue-adhesive glue and kept hydrated with a mixture of PBS and lubricant gel. Because of the thickness of the intestinal muscularis, the lumen interior was exposed by micro-incision along the intestinal wall followed by careful removal of large faecal matter and mounting on the customized stage. In some preparations, labelled bacteria were injected directly into the intestinal loop; otherwise, SPF mice were given 3 mM HADA in 250 µl by oral gavage at least 4 h before imaging. For delineation of the intestinal epithelial layer, the exposed luminal section was incubated in wheat germ agglutinin-AF633 (Life Technologies/ThermoFisher; 0.5 mg ml^{-1} in PBS) for 10 min to counterstain before imaging. Two-photon imaging was performed on an Ultima Two-Photon Microscope (Prairie Technologies/Bruker) equipped with a Tsunami Ti:sapphire laser with a 10 W MilleniaXs pump laser (Spectra-Physics) and a ×20/0.95 NA water-immersion objective (Olympus). The two-photon excitation wavelength was set to 815 nm for optimal fluorescence excitation of the HADA coumarin fluorophore while maintaining excitation of additional rhodamine green and TAMRA fluorophores for three-colour labelling. Fluorescence emission was detected with 665/65 nm, 590/50 nm, 525/50 nm and 450/50 nm bandpass filters for four-colour imaging. Raw image sequences were first processed with Volocity software (v.6.0, PerkinElmer). Autocontrasted images were processed with fine noise-reduction filters and each image channel was assigned a pseudo-colour according to emitted light wavelengths (bp665/65 nm, magenta; bp590/50 nm, red; bp525/50 nm, green; bp455/70 nm, blue). For time-lapse videos of the small intestine, processed image sequences were corrected for motion artefacts with ImageJ/Fiji (v.2.0.0). Images were motion-corrected by recursive registration of stacks/StackReg[34] with Affine transformation, followed by Elastic Stack Alignment[35] with Rigid transformation.

Statistical analyses. All statistical analyses were performed in Prism (GraphPad Software) and were based on normally distributed data sets with equal variance (Bartlett's test). Gender- and age-matched mice were randomly assigned to groups for *in vivo* experiments. Sample size was chosen for adequate numbers for statistical analyses. Investigators were not blinded during the experiments or outcome assessments. Where applicable, data points are presented as mean ± s.e.m. values unless otherwise stated. Data were inferred as statistically significant if P values were <0.05. The significance of differences between two groups was determined by two-tailed paired Student's t-test.

Data availability. The data that support the findings of this study are available from the corresponding author upon request, including, but not limited to, raw data, original images and further technical details.

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Author contributions

J.E.H. designed the experiments, analysed the data and wrote the manuscript with help from D.A. and A.S. D.A. provided expertise in two-photon intravital microscopy. D.L.K. supervised the study, edited the manuscript and provided helpful comments, with assistance from U.H.v.A.

Additional information

Supplementary information is available for this paper.

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Competing interests

The authors declare no competing financial interests.